Pergamon

www.elsevier.com/locate/neuroscience

PII: S0306-4522(00)00536-4

Neuroscience Vol. 103, No. 1, pp. 133–142, 2001

0306-4522/01 $20.00+0.00

THE POLYSIALYLATED NEURAL CELL ADHESION MOLECULE REACHES CELL SURFACES OF HYPOTHALAMIC NEURONS AND ASTROCYTES VIA THE CONSTITUTIVE PATHWAY

K. PIERRE, R. BONHOMME, B. DUPOUY, D. A. POULAIN and D. T. THEODOSIS*

INSERM U 378, Institut François Magendie, Université Victor Segalen Bordeaux II, 1 Rue Camille Saint Saens, F 33077 Bordeaux, France

Abstract—Understanding how neurons and glia sort and deliver cell adhesion molecules to their cell surface should provide important clues as to how such molecules participate in dynamic neuronal functions in the developing and adult brain. The present study examines translocation of polysialylated neural cell adhesion molecule (PSA-NCAM), a negative regulator of cell adhesion, in cells of the rat hypothalamo-neurohypophysial system in which it is expressed throughout life and which undergo morphological remodelling in response to stimulation. PSA-NCAM expression in this system does not vary markedly in relation to different conditions of regulated neurosecretion, suggesting that the glycoprotein reaches cell surfaces via the constitutive pathway. To study this more directly, we here used immunofluorescence for PSA on NCAM in live, unpermeabilized cells to monitor PSA-NCAM surface expression in organotypic slice cultures from postnatal rat hypothalami. Subsequent immunolabelling for oxytocin confirmed that the cultures included magnocellular oxytocinergic neurons displaying many properties of adult neurosecretory neurons *in situ*.

In the cultures, immunoreaction for PSA-NCAM was visible on the surface of oxytocinergic and non-oxytocinergic axons. This reaction disappeared after exposure of the cultures to endoneuraminidase, an enzyme which specifically cleaves α-2-8-linked PSA from NCAM. PSA-NCAM reappeared on axonal surfaces 4 h after enzyme washout. Such reexpression was visibly not affected by neuronal activity inhibition (blockade of Ca^{2+} channels with Mn^{2+}, of Na^+ channels with tetrodotoxin, or of glutamate receptors with 6-cyano-7-nitroquinoxaline-2,3-dione or D-2-amino-5-phosphonopentanoic acid) or facilitation (K^+ depolarization or GABA-A receptor blockade with bicuculline). In contrast, PSA-NCAM surface translocation was inhibited reversibly by cooling the cultures at 20°C, a procedure which blocks constitutive secretion and which resulted in accumulation of PSA-NCAM in the cytoplasm of oxytocinergic and non-oxytocinergic neurons. This treatment also revealed PSA-NCAM in the cytoplasm of underlying astrocytes.

Our observations provide direct evidence that PSA-NCAM reaches the cell surface of hypothalamic neurons and astrocytes via the constitutive pathway, independently of Ca^{2+} entry and enhanced neuronal activity. Thus, PSA-NCAM in the hypothalamo-neurohypophysial system would be continuously available to permit its cells to undergo remodelling whenever the proper stimulus intervenes.

Key words: adhesion molecules, constitutive secretion, oxytocin neurons, astrocytes, organotypic slice cultures.

Due to its negative charge and large hydrated volume, polysialic acid (PSA) on the neural cell adhesion molecule (NCAM) greatly attenuates adhesion. PSA constitutes up to 30% of the highly sialylated NCAM isoform, PSA-NCAM, thus rendering this cell surface glycoprotein an excellent molecular candidate to intervene in dynamic cellular changes underlying cell migration, neuritic outgrowth and neuronal plasticity (reviewed in Refs 32 and 34). To further understand the function of PSA-NCAM in the brain, one approach is to analyse the mechanisms by which it reaches the cell surface, in the different cells in which it is expressed. Such studies have provided conflicting observations so far since PSA-NCAM was shown to reach cell surfaces of cultured cells of neuroendocrine[1] or tumor[36] origin via the constitutive pathway and in cultured neurons and endocrine cells via the activity-dependent regulated pathway.[21,27]

In the present study, we examined the influence of neuronal activity on PSA-NCAM expression in a system which has served often to analyse regulated neurosecretion, namely, the hypothalamo-neurohypophysial system that secretes the neurohormones oxytocin and vasopressin (for a review see Ref. 15). The hypothalamo-neurohypophysial system expresses high levels of PSA-NCAM throughout life,[5,22,28,39,43] an expression which is essential to its capacity to undergo morphological remodelling in response to physiological stimulation.[39] In our earlier studies,[5,39,43] we reported that PSA-NCAM expression in the adult hypothalamo-neurohypophysial system, evaluated by

*Corresponding author. Tel.: +33-5-57573731; fax: +33-5-57573750.
E-mail address: dionysia.theodosis@bordeaux.inserm.fr (D. T. Theodosis).

Abbreviations: AP5, D-2-amino-5-phosphonopentanoic acid; CNQX, 6-cyano-7-nitroquinoxaline-2,3-dione; FITC, fluorescein thiocyanate; endoN, endoneuraminidase; GAP 43, growth associated protein; GFAP, glial fibrillary acidic protein; Ig, immunoglobulin; NCAM, neural cell adhesion molecule; OT-Np, oxytocin-related neurophysin; PBS, phosphate-buffered saline; PSA, polysialic acid; PSA-NCAM, polysialylated neural cell adhesion molecule; SON, supraoptic nucleus; TGN, trans Golgi network; TTX, tetrodotoxin.

immunocytochemistry and immunoblot analysis, did not vary significantly in relation to physiological conditions known to modulate its biosynthetic and secretory activities. Indeed, expression of PSA-NCAM differs markedly from that characterizing the neurohypopysial hormones or molecules like F3/contactin,[41] another cell adhesion molecule of the immunoglobulin (Ig) superfamily made by magnocellular neurons[29,31] and which follow the activity-dependent patterns of biosynthesis and transport characterizing molecules packaged in neurosecretory vesicles (for a review see Ref. 7).

To examine more directly the influence of neuronal activity on PSA-NCAM expression in hypothalamo-neurohypophysial system neurons and glia, we here used an *in vitro* model of the system, namely, organotypic slice cultures obtained from postnatal rat hypothalami. Earlier observations have established that these cultures are enriched in neurons secreting oxytocin, with morphological, electrophysiological and secretory properties similar to those of adult cells *in vivo*.[16,18–20] Moreover, the hypothalamic slices from which the cultures derive include inhibitory and excitatory afferent inputs controlling the activity of the neurons.[18] A major advantage of such cultures is that they allow us to visualize neurons in their entirety (see also Ref. 17). In the present experiments, therefore, double immunofluorescence for PSA on NCAM and for OT-related neurophysin (OT-Np) made it possible to monitor delivery of PSA-NCAM over the entire surface of individual, identified, live cells. This was further facilitated by prior treatment with endoneuraminidase (endoN), an enzyme which does not penetrate into cells and which specifically cleaves α-2-8-linked PSA residues from the extracellular domain of NCAM, without altering its backbone.[45]

EXPERIMENTAL PROCEDURES

The experiments were carried out on organotypic cultures obtained from slices of postnatal hypothalamus which included the supraoptic (SON) nuclei. The animals, six day-old male or female Wistar rat pups, were born and raised in the animal facilities of our Institute and were maintained under controlled temperature and light conditions. The cultures were prepared according to the roller tube method of Gähwiler, using a procedure described in detail in Ref. 20. All efforts were made to minimize the suffering and number of animals used and all our experiments conformed to local and international guidelines on the ethical uses of animals.

Visualization of polysialylated neural cell adhesion molecule

To visualize PSA-NCAM in the cultures, we used standard immunofluorescence techniques, according to procedures described in detail in our earlier studies.[5,18] Briefly, for single immunolabelling, after fixation in 4% paraformaldehyde and 0.15% picric acid in phosphate-buffered saline (PBS, 2 h, room temperature), the cultures were first treated with casein (0.5% in PBS) for 1 h to block non-specific sites, and then incubated in an antibody which specifically recognises PSA on NCAM (kindly provided by G. Rougon); its production and characterization are described in detail in Ref. 33. It is a mouse monoclonal IgM recognising specifically α-2,8-linked PSA with chain length greater than 12 residues. The virtual absence of PSA in NCAM-deficient mice has provided the most compelling evidence that NCAM is indeed the major carrier of PSA in the CNS.[10,30] The antibody was used at a dilution of 1/4000–1/6000 and the cultures were incubated for 24–48 h at 4°C. For double immunolabelling, they were incubated for 48 h at 4°C in mixtures of primary antibodies containing anti-PSA-NCAM (diluted 1/4000) and mouse monoclonal IgGs raised against oxytocin-related neurophysin (OT-Np, diluted 1/400, kindly provided by H. Gainer; see Ref. 4 for its production and specificity). In a few cases, the anti-OT-Np antibodies were replaced by mouse monoclonal anti-glial fibrillary acidic protein (GFAP, diluted 1/500, Sigma, France), recognising astrocytes, or the axonal marker,[35] anti-neuronal growth associated protein-43 (GAP-43, diluted 1/500, Boehringer Mannheim France). All primary antibodies were diluted in Tris-buffered saline containing 0.25% bovine serum albumin. Rat fluorescein thiocyanate (FITC)-conjugated anti-mouse IgM Igs (diluted 1/500, Immunotech, France) served to identify PSA immunoreactivity and goat anti-mouse IgGs conjugated with Texas Red (diluted 1/500, Biosys, France) OT-Np, GFAP or GAP-43 immunoreactivities. Controls included omitting primary antibodies or substitution by diluted mouse ascites fluid containing IgM irrelevant antibodies. No specific staining was visible on these preparations.

The cultures were mounted with fluoromount (Vectashield, Vector Laboratories) and examined with standard epifluorescence (Leica DMRB) or confocal microscopy (Inverted CSLM System, Molecular Dynamics, Sunnyvale, CA) with appropriate filters. For the latter, serial optical sections were collected (1 μm/step; pixel size 0.11 μm); each section was examined individually or was used to generate projections.

Effect of neuronal activity

Cultures, at least three weeks old, were first incubated with endoN diluted 1/10000 from a stock solution containing 1 mg protein/ml. The activity of the enzyme was titrated to be 3500 U/mg. The enzyme had been purified from phage K1;[46] it degrades rapidly and specifically linear polymers of sialic acid with α-2,8-linkage with a minimum length of seven to nine residues, characteristic of sialic acid residues associated with NCAM.[13] After a 12 h incubation, PSA-NCAM was no longer visible on cell surfaces, as judged by immunohistochemistry for PSA (see Fig. 2).

To study surface reexpression of the glycoprotein (in 26 independent experiments), the cultures were carefully rinsed of enzyme, and then incubated for 2–8 h, at 37°C, in culture medium that included anti-PSA-NCAM. The antibody was added alone (diluted 1/4000; $n = 47$ cultures) or together with factors which affect Ca^{2+}entry (4 μM Mn^{2+}; $n = 13$)[6] or neuronal activity, such as 0.25 μM tetrodotoxin (TTX, Sigma; $n = 9$), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX) and D-2-amino-5-phosphonopentanoic acid (AP5; Sigma; 20 and 50 μM, respectively; $n = 5$), 56 mM KCl ($n = 14$), or 10 μM bicuculline (Sigma; $n = 5$). After careful rinsing, all cultures were fixed in freshly prepared 4% paraformaldehyde and 0.15% picric acid in 0.1 M sodium phosphate buffer (2 h, room temperature). They were then incubated with anti-OT-Np antibodies. Double immunofluorescence was used to visualize immunoreactivities.

Effect of temperature

A temperature-induced transport block was produced by maintaining the preparations at 20°C (see also Refs 24 and 26). This was carried out on cultures that had been exposed previously to endoN for 12 h, as described above. After enzyme washout, the cultures were left for 24 h in a room kept at 20°C ($n = 9$, in six independent experiments). Following careful rinsing, some were fixed in 4% paraformaldehyde and 0.15% picric acid (2 h, room temperature). Another group was brought back to 37°C for 4–7 h after cooling and then fixed. All cultures were then incubated in combinations of antibodies that included anti-PSA and anti-OT-Np or anti-GFAP antibodies; immunoreactivities were revealed by immunofluorescence.

RESULTS

As we have reported earlier,[18,20] hypothalamic neurons

and glia differentiate well in our organotypic slice cultures in which they can be maintained for relatively long periods of time (over one month). By three weeks *in vitro*, the slices thin out and this allows us to visualize neurons in their entirety. Immunolabelling with antibodies against OT-Np reveals many oxytocinergic neurons, occuring in clusters (Fig. 1A) and displaying morphological characteristics of OT neurons *in situ* (Figs 1A, D, 4A). In the present study, double immunofluorescence revealed a rich PSA immunolabeling in these cultures, limited to surfaces of axonal-like fibers of some neurons, that were or not oxytocinergic (Fig. 1C1, D1, E1). Unlike dendritic-like neurites, such fibers had regular diameters, and displayed dilatations of various sizes along their length (Fig. 1B–E). The axonal-like nature of the PSA-positive fibers was confirmed in some cultures by their immunoreaction for GAP-43[35] (Fig. 1E).

Light and confocal microscopy showed that PSA immunoreactivity occured in a discontinuous manner on the labelled axonal surfaces (Fig. 1C1, D1, E1). Not all oxytocinergic axons displayed PSA immunoreactivity (Fig. 1C1). PSA immunolabel was not detected intracellularly, nor on the surface of somatic or dendritic profiles (Fig. 1D, D1).

To follow the appearance of newly formed PSA-NCAM on cell surfaces, we first removed preexisting PSA on NCAM with endoN and then monitored its reappearance with immunofluorescence in live cultures at different times following enzyme washout. After a 12 h exposure to endoN, PSA immunoreaction was no longer detectable on any surface in the cultures (Fig. 2A1). The enzyme treatment, even when prolonged for 24 h, did not affect the viability of the cultured cells since there was no evidence of cell swelling or death. PSA immunoreactivity became visible again on the surfaces of some axons 4 h after enzyme washout (Fig. 2B1) and by 7 h, many fibers, oxytocinergic or not, displayed PSA-immunoreactivity on their surfaces (Fig. 2C1).

The reappearance of PSA immunoreactivity on axonal surfaces after endoN treatment of the cultures appeared visibly unaltered when they were exposed to factors known to affect neuronal activity. Thus, Mn^{2+} blockade of Ca^{2+} entry[14] (Fig. 3A1) or blockade of voltage-dependent Na^+ channels with TTX (Fig. 3B1) did not affect PSA-NCAM surface reexpression so that PSA immunoreactivity was visible on axonal fibers 4 h after enzyme washout. Likewise, facilitation of electrical activity by K^+ depolarization (Fig. 3C1) resulted in PSA surface immunolabelling similar to that in control cultures. Our earlier observations[18,19] have established that the electrical activity of OT neurons in these cultures, as *in vivo*, is controlled essentially by GABA and glutamate synaptic inputs. In the present experiments, we therefore inhibited all glutamate activity with the antagonists, CNQX and 5-AP, yet this did not visibly affect reappearance of PSA immunoreactivity on axonal surfaces after endoN washout. Likewise, blockade of inhibitory inputs with the GABA-A antagonist, bicuculline, had no effect on the reappearance of PSA immunoreactivity.

In contrast, reexpression of PSA-NCAM on axonal surfaces after endoN treatment was completely blocked in cultures cooled to 20°C for 24 h. Such cooling is known to arrest the constitutive pathway of delivery of glycoproteins destined for the cell surface by blocking them in the trans Golgi network (TGN; reviewed in Refs 24 and 26). In the cooled cultures, we were thus unable to detect any PSA immunoreactivity on any axonal surface with light or confocal microscopy. However, it heavily filled the cytoplasm of neuronal somata (Fig. 4 A). In accord with earlier observations,[24,26] we found that the effect of cooling was reversible since PSA immunoreactivity disappeared progressively from the cytoplasm of neuronal somata (Fig. 5A1) and began to appear on axonal surfaces (Fig. 5B1) within 4 h of warming the cultures to 37°C. It is noteworthy that it is difficult to detect PSA immunoreaction in underlying glia in normal cultures (Figs 1–3) yet after cooling, a variable amount of reaction was visible in the somata and processes of these cells as well (Fig. 4B).

DISCUSSION

As *in vivo*,[5,22,28,39,43] magnocellular neurons maintained in organotypic slice cultures obtained from postnatal rat hypothalamus express high levels of PSA-NCAM. Since our *in vitro* preparations made it possible to react anti-PSA antibodies directly with live cells, and since we could visualize the cultured neurons in their entirety, we were able to establish that PSA-NCAM cell surface expression in these cells is polarized to their axons, in the presence or absence of stimulation. This agrees with earlier morphological and immunoblot observations showing that there is much PSA-NCAM in the neurohypophysis, composed essentially of neurosecretory axons and terminals, regardless of the condition of the animal.[22,39,43] Moreover, our present data are in agreement with electron microscopic observations that revealed strong PSA-NCAM immunoreactivity on the surface of neurosecretory axons in the SON[39] and in the neurohypophysis.[22,39,43]

Biochemical studies of tumoral and developing cells established that polysialylation of NCAM isoforms is a relatively rapid process[1] that occurs in the TGN[1,36] via specific polysialyltransferases.[2,3,23] A similar process must occur in hypothalamo-neurohypophysial system cells in which these enzymes have been detected.[37] Like other proteins destined for the cell surface in eukaryotic cells, PSA-NCAM must then follow either of two pathways to reach cell surfaces (reviewed in Ref. 8), the regulated pathway, whereby, packaged and transported in secretory vesicles, it would be released when the cell is stimulated by a secretagogue or increased activity, or the constitutive pathway, whereby packaged in vesicles, it would be released shortly after synthesis. From our present observations, it is clear that PSA-NCAM follows the latter pathway in the cells of the hypothalamo-neurohypophysial system and arrives at axonal surfaces within a few hours.

Unlike regulated secretion, constitutive secretion occurs quite independently of extracellular signals and is not closely dependent on electrical activity and enhanced

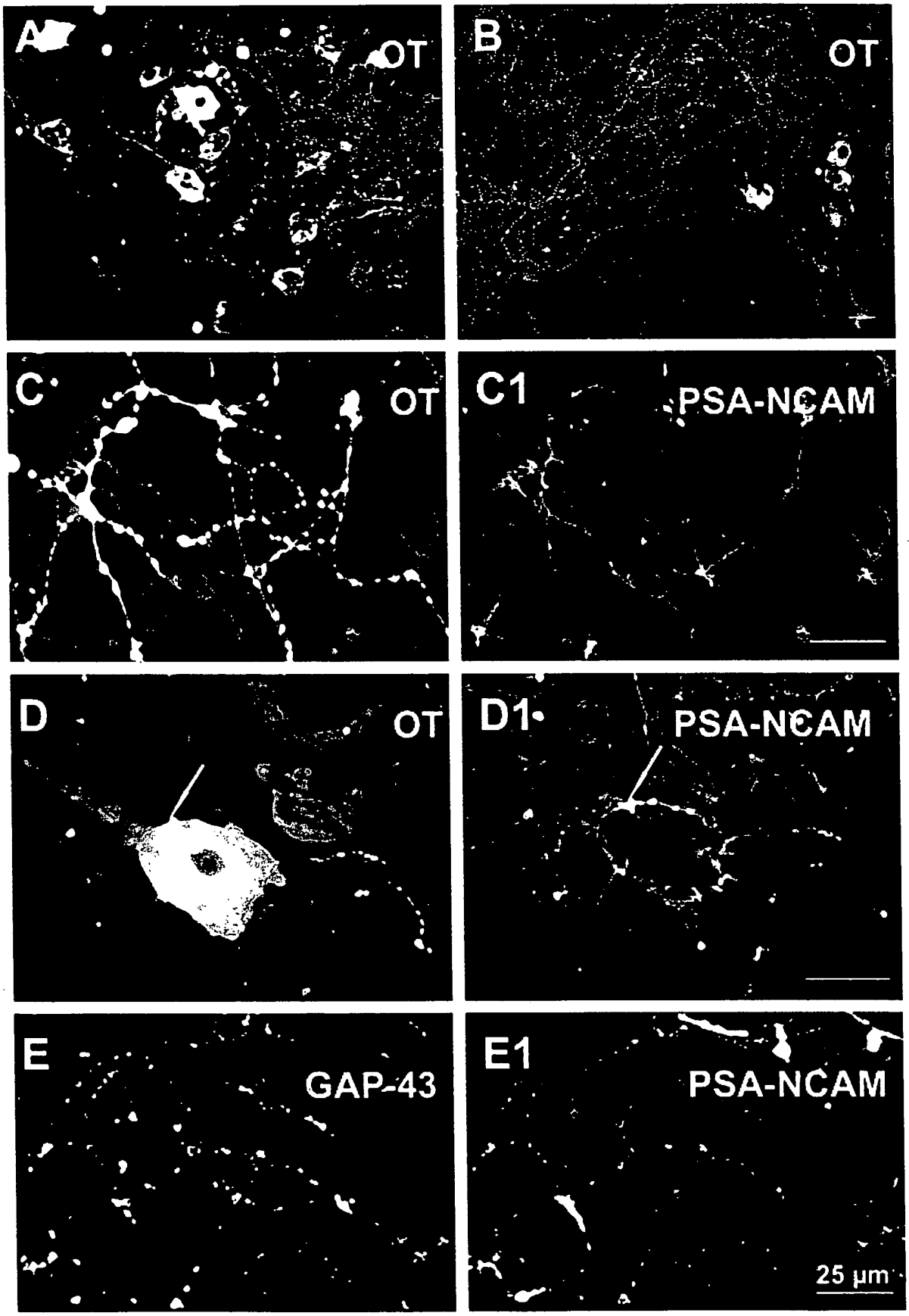

Fig. 1.

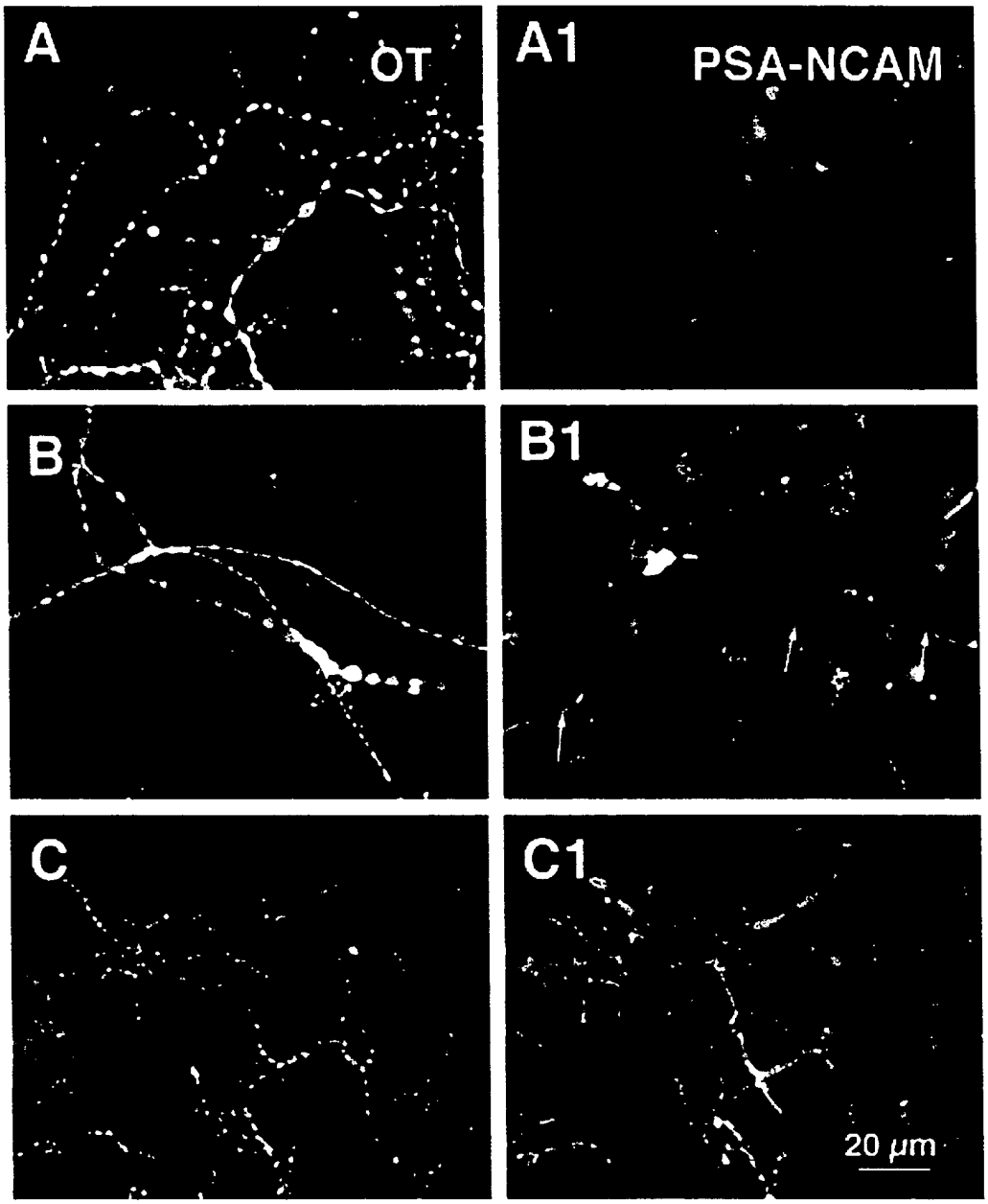

Fig. 2. Neuronal reexpression of PSA-NCAM monitored by immunofluorescence for PSA after endoneuraminidase (endoN) treatment of hypothalamic organotypic slice cultures. After enzyme washout, live cells were maintained in culture medium containing PSA antibody; they were then fixed and incubated with OT-Np antibodies. Immunoreactivities were revealed with appropriate fluorescent labels. Such immunolabelling for OT-Np (A–C) and PSA (A1–C1) shows that after overnight exposure to the enzyme, PSA immunolabel is no longer detectable on the surface of oxytocinergic axons (A, A1). The fluorescence, still visible in Fig. A1, is due to background labelling. Specific PSA immunoreaction reappears 4 h after enzyme washout (arrows, B1); by 7 h (C1), it covers the surface of many axons. Note that OT-Np immunoreactivity is not affected by this treatment. Epifluorescence with appropriate filters. Scale bar = 20 μm.

Fig. 1. (A, B) Magnocellular oxytocinergic (OT) neurons maintained in hypothalamic organotypic slice cultures. After three weeks *in vitro*, the neurons, identified by immunofluorescence for oxytocin-related neurophysin (OT-Np), possess large (20–40 μm) somata from which arise two to four thick, dendritic-like processes; their axons are thin, beaded and display complex arborization patterns (B). (C–E) Expression of the polysialylated isoform of neural cell adhesion molecule (PSA-NCAM), in hypothalamic organotypic slice cultures. After three weeks *in vitro*, immunoreactivity for PSA on NCAM is visible on the surface of axonal-like fibers that are (C, C1) or not (D, D1) oxytocinergic. In D, D1, PSA-positive fibers (arrows) appear to contact an OT soma. Note that PSA immunoreactivity is not associated with the surface of OT somata and dendrites. In E, E1, the axonal nature of fibers displaying PSA immunoreactivity is illustrated by their reaction for the axonal phosphoprotein, GAP-43. Epifluorescence with appropriate filters. Scale bars = 50 μm (A, B, D, D1) and 25 μm (C, C1, E, E1).

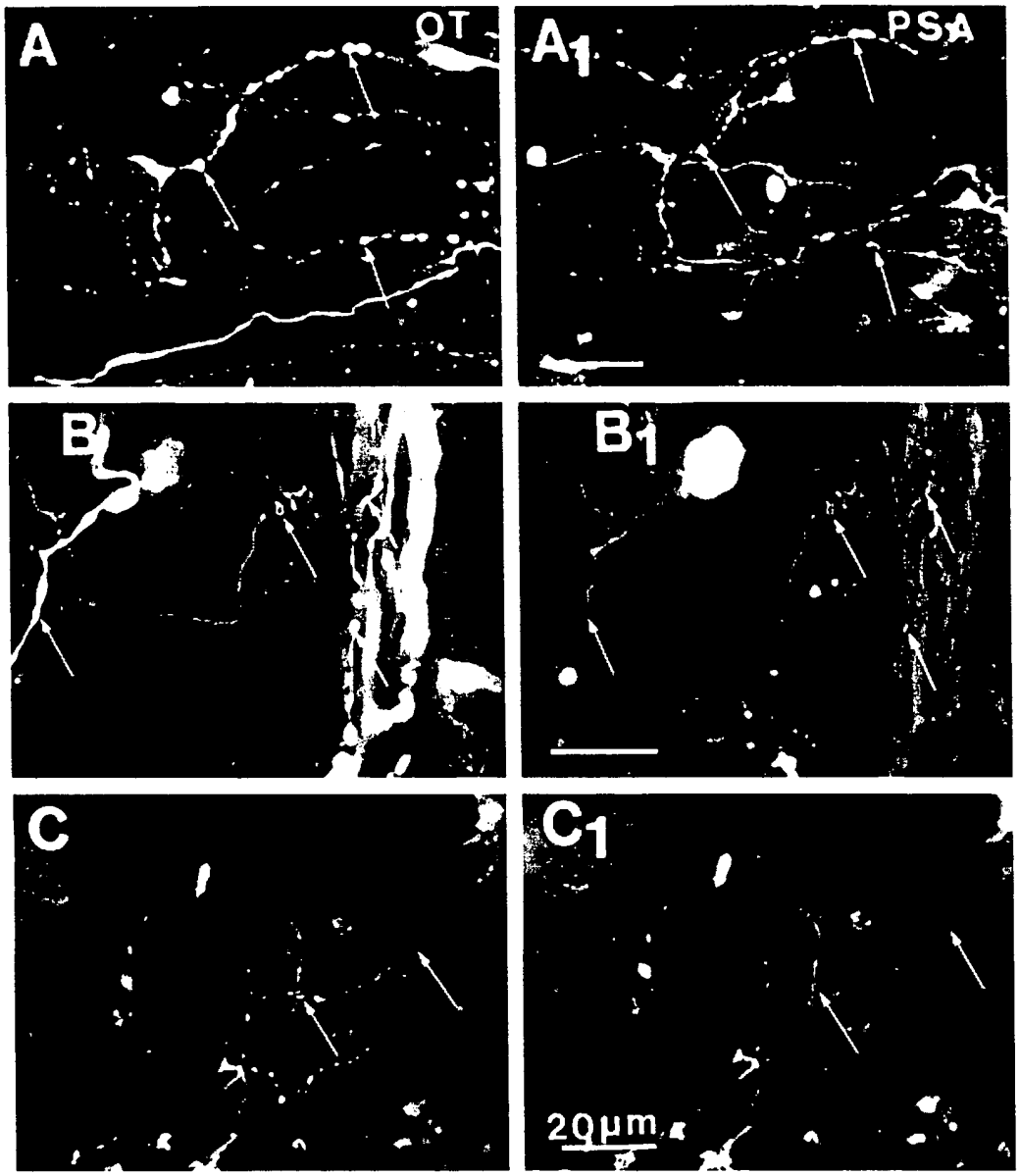

Fig. 3. Effect of neuronal activity on PSA-NCAM reexpression after endoN treatment in hypothalamic organotypic slice cultures. After enzyme washout, live cells were maintained in culture medium containing PSA antibody and factors affecting neuronal activity; they were then fixed and incubated with OT-Np antibodies. Four hours after enzyme washout, incubation of cultures with medium containing Mn^{2+} to block Ca^{2+} entry (A, A1) or TTX to inhibit electrical activity (B, B1) had no effect and PSA immunolabel (arrows) was visible on OT axons. Likewise, cultures incubated in 56 mM KCl (C, C1) displayed PSA immunoreactivity on axonal surfaces in a manner similar to that in cultures incubated in normal medium. Note that the highly fluorescent circular profiles in all figures are due to unspecific background labelling. Epifluorescence with appropriate filters. Scale bars = 20 µm.

entry of Ca^{2+} after depolarization (reviewed in Refs 8 and 9). Our organotypic cultures allowed us to test directly the role of electrical activity on PSA-NCAM surface expression since their magnocellular neurons retain electrical properties of hypothalamo-neurohypophysial neurons *in vivo*, including their control by glutamatergic and GABAergic afferents.[18–20] In agreement with the tenet that PSA-NCAM follows a constitutive pathway in these cells, we found that all procedures known to alter their electrical activity or to couple electrical activity with neurosecretion failed to modify expression of PSA-NCAM on their cell surface. Thus, blockade of electrical activity by TTX, of synaptic drive by glutamate and GABA receptor antagonists, or of Ca^{2+} entry by Ca^{2+} channel blockers, did not change the time-course of PSA-NCAM reexpression after enzymatic removal of all surface PSA. However, stimulation of electrical activity by elevated extracellular K+ concentrations known to facilitate depolarization and neurosecretion[19] failed to accelerate reexpression. Taken together, then, all these observations make it clear that PSA-NCAM expression in hypothalamo-neurohypophysial neurons does not depend on their electrical activity.

In contrast, the data obtained after cooling strongly

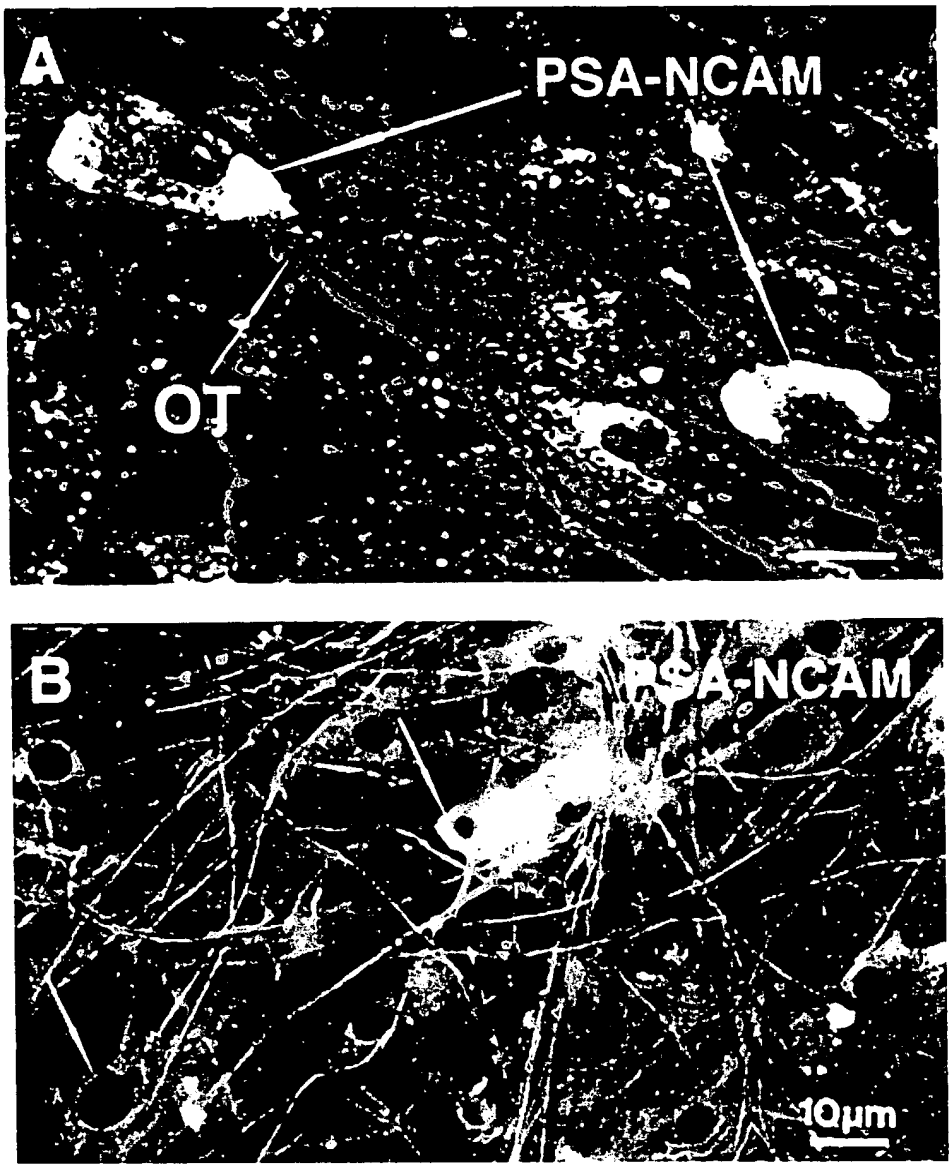

Fig. 4. PSA immunoreactivity in hypothalamic organotypic cultures maintained at 20°C. Incubation of cultures at 20°C for 12 h resulted in accumulation of PSA immunolabeling (green–yellow) in the cytoplasm of OT-Np- (red, A) and GFAP-positive astrocytic (arrows, B) somata. PSA immunoreactivity is not detectable on axonal surfaces (A). Note that such cooling did not affect peptide transport since OT-Np immunoreactivity (A) is clearly visible in axons and dendrites. (A) Confocal image generated from an overlay of 20 optical sections of two single projections for PSA (green) or OT-Np (red). (B) Epifluorescence with appropriate filters. Scale bars = 30 μm.

support the contention of a constitutive pathway of PSA-NCAM expression. Cooling down to 20°C, a procedure known to retard and even block the constitutive pathway of glycoprotein transport,[9,24,26,44] blocked surface expression of PSA-NCAM in our neurons, as it was shown to do in other cells.[1,36] It is noteworthy that OT-Np immunoreactivity in oxytocinergic axons and dendrites in the cooled cultures was not affected by this temperature, which is not surprising since regulated transport continues unaffected down to 4°C.[9,26,44] As expected,[24,26] the effect of cooling to 20°C was reversed by warming the cultures to 37°C. Similar observations can be obtained in acute slices of adult rat hypothalamus where accumulation of PSA immunoreactivity is detectable in the somata of some magnocellular neurons maintained at 18°C even for 4 h (Theodosis & Israel, unpublished observations).

That delivery of PSA-NCAM to the plasmalemma of neurosecretory axons occurs via the constitutive pathway means that upon exiting the TGN, PSA-NCAM is not sequestered in neurosecretory granules.[44] This explains why PSA immunoreactivity is not visible in neurosecretory granules[22,39] and why PSA-NCAM expression in the hypothalamo-neurohypophysial system is not affected by situations which modify neurosecretion.[5,39,43] For example, during lactation and osmotic stimulation, levels of neurohypophysial hormones significantly increase in magnocellular somata and decrease in

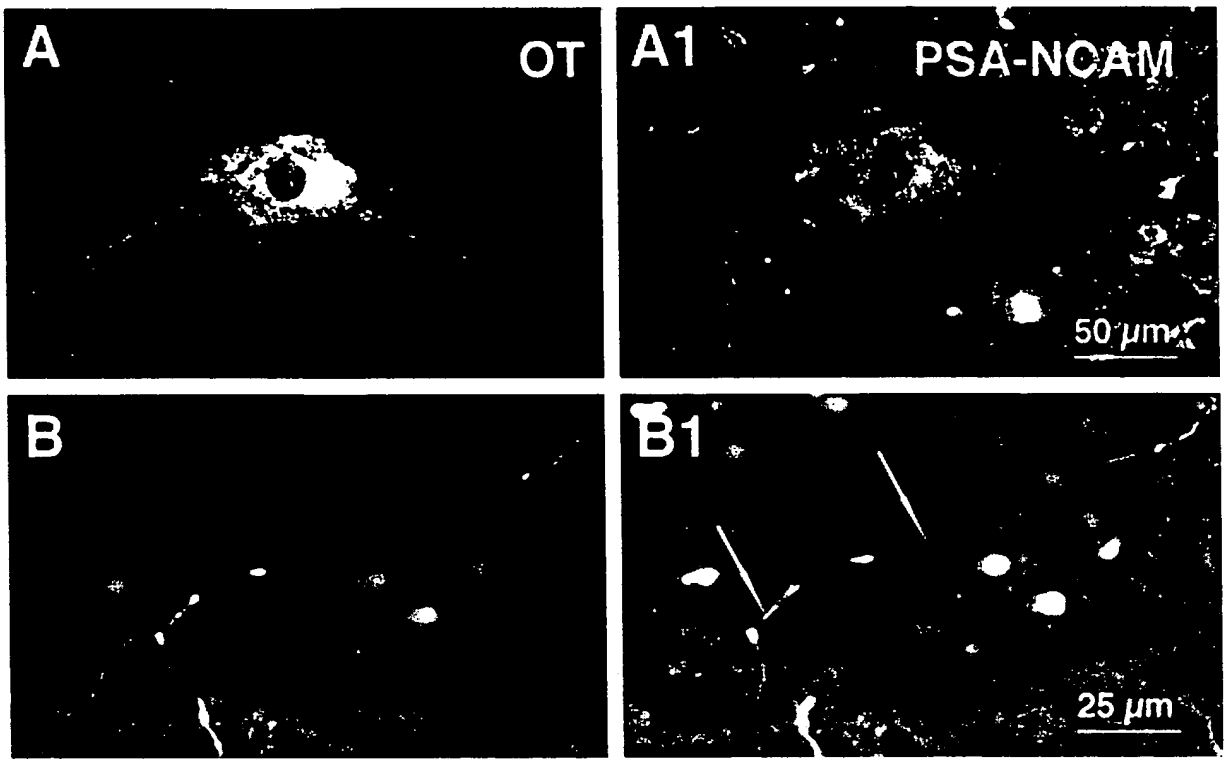

Fig. 5. PSA immunoreactivity in hypothalamic organotypic cultures treated with endoN for 12 h, then incubated at 20°C for 24 h, and again at 37°C for 4 h. The cooling effect is reversible since PSA immmunoreactivity, while still visible in the cytoplasm of OT somata (A1), now also appears on the surface (arrows, B1) of OT axonal fibers (B). Epifluorescence with appropriate filters. Scale bars = 50 μm (A, A1) and 25 μm (B, B1).

neurohypophysial axons from which they are released by regulated exocytosis. The expression of molecules colocalized with these peptides, like chromogranins[11] or F3/contactin,[31] another cell adhesion molecule of the Ig superfamily, follow similar patterns. In comparison, the expression of PSA-NCAM appears relatively stable.[41]

Not only neurons, but astrocytes express PSA-NCAM in the hypothalamo-neurohypophysial system as well. This is particularly visible *in vivo* where strong PSA immunoreactivity is associated with astrocytes in the hypothalamic magnocellular nuclei[39,43] and pituicytes, the astrocytic derivatives of the neurohypophysis.[22,39,43] In our organotypic cultures, however, PSA-NCAM levels in astrocytes must be low since we were not able to detect PSA immunoreactivity in these cells under normal or stimulated conditions of neuronal activity. However, the cells were strongly PSA-immunopositive when they were maintained at 18–20°C. Astrocytes synthesize and release a large variety of substances,[25] but relatively little attention has been paid to the mechanisms by which they are transported. Although one would expect that glia possess a constitutive mechanism for glycoprotein transport to their cell surface, it is not clear whether they can also undergo activity-dependent regulated secretion (see also Ref. 25). Concerning hypothalamo-neurohypophysial astrocytes, there is no morphological evidence that they possess recognisable secretory vesicles or release zones.[38] As shown here, transport of molecules like PSA-NCAM to the cell surface of hypothalamic astrocytes occurs via the constitutive pathway.

Earlier studies reported that PSA-NCAM reaches the cell surface via the constitutive[1,36] or regulated pathway.[21,27] Taken together with our present observations, this would mean that this particular cell adhesion molecule can follow different pathways for surface expression in different cells. This possibility is not unlikely in view of recent observations showing that glycoproteins like brain derived neurotrophic factor and neurotrophin 3 can follow the constitutive and regulated pathways within the same cell and can even be shunted from one pathway to the other under certain conditions.[12]

CONCLUSION

Neurons and glia of the hypothalamo-neurohypophysial system undergo significant activity-dependent changes in their morphology in the adult (reviewed in Ref. 42). Using *in vivo* enzymatic manipulation of PSA-NCAM expression, we recently provided direct evidence that PSA on NCAM is indeed necessary for such plasticity since neuronal and glial remodelling was inhibited in the endoN-treated SON of lactating and dehydrated rats.[39] That PSA-NCAM is synthetized by both neurons and astrocytes of this neurosecretory system and that it is constitutively expressed on their surfaces would mean then that it is constantly available to promote such cellular changes. As we have proposed earlier (see Ref. 40), it is highly probable that PSA-NCAM is not an inductive factor for such changes, but rather, a permissive factor

necessary to allow the cells to undergo remodelling whenever the proper stimulus intervenes.

Acknowledgements—We are grateful to Dr G. Rougon for her generous gift of PSA antibodies and enzyme preparation and to Dr H. Gainer for his generous gift of OT-Np antibodies. We also thank Mme I. Svahn (SERCOMI, Université Victor Segalen Bordeaux II) for her expert photographic work.

REFERENCES

1. Alcaraz G. and Goridis C. (1991) Biosynthesis and processing of polysialylated NCAM by AtT-20 cells. *Eur. J. Cell Biol.* **55,** 165–173.
2. Angata K., Nakayama J., Fredette B., Chong K., Ranscht B. and Fukuda M. (1997) Human STX polysialyltransferase forms the embryonic form of the neural cell adhesion molecule. Tissue-specific expression, neurite outgrowth, and chromosomal localization in comparison with another polysialyltransferase, PST. *J. biol. Chem.* **272,** 7182–7190.
3. Angata K., Suzuki M. and Fukuda M. (1998) Differential and cooperative polysialylation of the neural cell adhesion molecule by two polysialyltransferases, PST and STX. *J. biol. Chem.* **273,** 28,524–28,532.
4. Ben-Barak Y., Russell J. T., Whitnall M. H., Ozato K. and Gainer H. (1985) Neurophysin in the hypothalamo-neurohypophysial system: I. production and characterization of monoclonal antibodies. *J. Neurosci.* **5,** 81–97.
5. Bonfanti L., Olive S., Poulain D. A. and Theodosis D. T. (1992) Mapping of the distribution of polysialylated neural cell adhesion molecule throughout the central nervous system of the adult rat: an immunohistochemical study. *Neuroscience* **49,** 419–436.
6. Bourque C. W. and Renaud L. P. (1985) Calcium-dependent action potentials in rat supraoptic neurosecretory neurones recorded *in vitro*. *J. Physiol.* **363,** 419–428.
7. Brownstein M. J., Russell J. T. and Gainer H. (1980) Synthesis, transport, and release of posterior pituitary hormones. *Science* **207,** 373–378.
8. Burgess T. L. and Kelly R. B. (1987) Constitutive and regulated secretion of proteins. *A. Rev. Cell Biol.* **3,** 243–293.
9. Burgoyne R. D. and Morgan A. (1993) Regulated exocytosis. *Biochem. J.* **293,** 305–316.
10. Cremer H., Lange R., Christoph A., Plomann M., Vopper G., Roes J., Brown R., Baldwin S., Kraemer P., Scheff S., Barthels D., Rajewsky K. and Wille W. (1994) Inactivation of the N-CAM gene in mice results in size reduction of the olfactory bulb and deficits in spatial learning. *Nature* **367,** 455–459.
11. El Majdoubi M., Metz-Boutigue M.-H., Garcia-Sablonne P., Theodosis D. T. and Aunis D. (1996) Immunocytochemical localization of chromogranin A in the hypothalamo-neurohypophysial system of the rat. *J. Neurocytol.* **25,** 405–416.
12. Farhadi H. F., Mowla S. J., Petrecca K., Morris S. J., Seidah N. G. and Murphy R. A. (2000) Neurotrophin-3 sorts to the constitutive secretory pathway of hippocampal neurons and is diverted to the regulated secretory pathway by coexpression with brain-derived neurotrophic factor. *J. Neurosci.* **20,** 4059–4068.
13. Finne J. and Mäkelä P. H. (1985) Cleavage of the polysialosyl units of brain glycoproteins by a bacteriophage endosialidase. *J. biol. Chem.* **260,** 1265–1270.
14. Fisher T. E. and Bourque C. W. (1995) Voltage-gated calcium currents in the magnocellular neurosecretory cells of the rat supraoptic nucleus. *J. Physiol.* **486,** 571–580.
15. Gainer H. and Wray S. (1994) Cellular and molecular biology of oxytocin and vasopressin. In *The Physiology of Reproduction* (eds Knobil E. and Neill J. D.), Second Edition. Raven, New York.
16. Gähwiler B. H. (1988) Organotypic culture of neuronal tissue. *Trends Neurosci.* **11,** 484–489.
17. Gähwiler B. H., Capogna M., Debanne D., McKinney R. A. and Thompson S. M. (1997) Organotypic slice cultures: a technique has come of age. *Trends Neurosci.* **20,** 471–477.
18. Jourdain P., Dupouy B., Bonhomme R., Poulain D. A., Israel J. M. and Theodosis D. T. (1999) Visualization of local afferent inputs to magnocellular oxytocin neurons *in vitro*. *Eur. J. Neurosci.* **11,** 1–13.
19. Jourdain P., Israel J. M., Dupouy B., Oliet S. H. R., Allard M., Vitiello S., Theodosis D. T. and Poulain D. A. (1998) Evidence for a hypothalamic oxytocin-sensitive pattern-generating network governing oxytocin neurons *in vitro*. *J. Neurosci.* **18,** 6641–6649.
20. Jourdain P., Poulain D. A., Theodosis D. T. and Israel J. M. (1996) Electrical properties of oxytocin neurons in organotypic cultures from postnatal rat hypothalamus. *J. Neurophysiol.* **76,** 2772–2785.
21. Kiss J. Z., Wang C., Olive S., Rougon G., Lang J., Baetens D., Harry D. and Pralong W. F. (1994) Activity-dependent mobilization of the adhesion molecule polysialic NCAM to the cell surface of neurons and endocrine cells. *Eur. molec. Biol. Org. J.* **13,** 5284–5292.
22. Kiss J. Z., Wang C. and Rougon G. (1993) Nerve-dependent expression of high polysialic acid neural cell adhesion molecule in neurohypophysial astrocytes of adult rats. *Neuroscience* **53,** 213–222.
23. Kojima N., Kono M., Yoshida Y., Tachida Y., Nakafuku M. and Tsuji S. (1996) Biosynthesis and expression of polysialic acid on the neural cell adhesion molecule is predominantly directed by ST8Sia/STX during *in vitro* neuronal differentiation. *J. biol. Chem.* **271,** 22,058–22,062.
24. Kuismanen E. and Saraste J. (1989) Low temperature-induced transport blocks as tools to manipulate membrane traffic. *Meth. Cell Biol.* **32,** 257–274.
25. Martin D. L. (1992) Synthesis and release of neuroactive substances by glial cells. *Glia* **5,** 81–94.
26. Matlin K. S. and Simons K. (1983) Reduced temperature prevents transfer of a membrane glycoprotein to the cell surface but does not prevent terminal glycosylation. *Cell* **34,** 233–243.
27. Muller D., Wang C., Skibo G., Toni N., Cremer H., Calaora V., Rougon G. and Kiss J. Z. (1996) PSA-NCAM is required for activity-induced synaptic plasticity. *Neuron* **17,** 413–422.
28. Nothias F., Vernier P., Von Boxberg Y., Mirman S. and Vincent J. D. (1997) Modulation of NCAM polysialylation is associated with morphofunctionnal modification in the hypothalamo-neurohypophysial system during lactation. *Eur. J. Neurosci.* **9,** 1553–1565.
29. Olive S., Rougon G., Pierre K. and Theodosis D. T. (1995) Expression of a glycosyl phosphatidylinositol-anchored adhesion molecule, the glycoprotein F3, in the adult rat hypothalamo-neurohypophysial system. *Brain Res.* **689,** 271–280.
30. Ono K., Tomasiewicz H., Magnuson T. and Rutishauser U. (1994) N-CAM mutation inhibits tangential neuronal migration and is phenocopied by enzymatic removal of polysialic acid. *Neuron* **13,** 595–609.
31. Pierre K., Rougon G., Allard M., Bonhomme R., Gennarini G., Poulain D. A. and Theodosis D. T. (1998) Regulated expression of the cell adhesion glycoprotein F3 in adult hypothalamic magnocellular neurons. *J. Neurosci.* **18,** 5333–5343.
32. Rougon G. (1993) Structure, metabolism and cell biology of polysialic acids. *Eur. J. Cell Biol.* **61,** 197–207.
33. Rougon G., Dubois C., Buckley N., Magnani J. L. and Zollinger W. (1986) A monoclonal antibody against meningococcus group B polysacharides distinguishes embryonic from adult N-CAM. *J. Cell Biol.* **103,** 2429–2437.
34. Rutishauser U. and Landmesser L. (1996) Polysialic acid in the vertebrate nervous system: a promoter of plasticity in cell-cell interactions. *Trends Neurosci.* **19,** 422–427.
35. Sargent P. B. (1989) What distinguishes axons from dendrites? Neurons know more than we do. *Trends Neurosci.* **12,** 203–205.

36. Scheidegger P., Papay J., Zuber C., Lackie P. M. and Roth J. (1994) Cellular site of synthesis and dynamics of cell surface re-expression of polysialic acid of the neural cell adhesion molecule. *Eur. J. Biochem.* **225,** 1097–1103.
37. Soares S., Von Boxberg Y., Ravaille-Veron M., Vincent J. D. and Nothias F. (2000) Morphofunctional plasticity in the adult hypothalamus induces regulation of polysialic acid-neural cell adhesion molecule through changing activity and expression levels of polysialyltransferases. *J. Neurosci.* **20,** (7) 2551–2557.
38. Theodosis D. T. (1979) Endocytosis in glial cells (pituicytes) of the rat neurohypophysis demonstrated by incorporation of horseradish peroxidase. *Neuroscience* **4,** 417–425.
39. Theodosis D. T., Bonhomme R., Vitiello S., Rougon G. and Poulain D. A. (1999) Cell surface expression of polysialic acid on NCAM is a prerequisite for activity-dependent morphological neuronal and glial plasticity. *J. Neurosci.* **19,** 10,228–10,236.
40. Theodosis D. T., El Majdoubi M., Pierre K. and Poulain D. A. (1998) Factors governing activity-dependent structural plasticity of the hypothalamo-neurohypophysial system. *Cell. molec. Neurobiol.* **18,** 285–298.
41. Theodosis D. T., Pierre K. and Poulain D. A. (2000) Differential expression of two adhesion molecules of the immunoglobulin superfamily, F3 and polysialylated NCAM, in the hypothalamic magnocellular neurones capable of plasticity. *Expl Physiol.* **85S,** 187S–196S.
42. Theodosis D. T. and Poulain D. A. (1993) Activity-dependent neuronal-glial and synaptic plasticity in the adult mammalian hypothalamus. *Neuroscience* **57,** 501–535.
43. Theodosis D. T., Rougon G. and Poulain D. A. (1991) Retention of embryonic features by an adult neuronal system capable of plasticity: Polysialylated N-CAM in the hypothalamo-neurohypophysial system. *Proc. natn. Acad. Sci. USA* **88,** 5494–5498.
44. Tooze S. A. and Huttner W. B. (1990) Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell* **60,** 837–847.
45. Vimr E. R., McCoy R. D., Vollger H. F., Wilkinson N. C. and Troy F. A. (1984) Use of prokaryotic-derived probes to identify polysialic acid in neonatal neuronal membranes. *Proc. natn. Acad. Sci. USA* **81,** 1971–1975.
46. Wang C., Rougon G. and Kiss J. Z. (1994) Requirement of polysialic acid for the migration of the O-2A glial progenitor cell from neurohypophysial explants. *J. Neurosci.* **14,** 4446–4457.

(*Accepted* 13 *November* 2000)